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FAB-MS characterization of sialyl Lewis^x determinants on polylactosamine chains of human airway mucins secreted by patients suffering from cystic fibrosis or chronic bronchitis

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Although a large body of structural data exists for bronchial mucins from cystic fibrosis (CF) and chronic bronchitis (CB) patients, little is known about terminal structures carried on poly-*N*-acetyllactosamine antennae. Such structures are of interest because they are potential ligands for bacterial adhesins and other lectins. In this study, we have used fast atom bombardment mass spectrometry (FAB-MS) to examine terminal sequences released by endo-β-galactosidase from *O*-glycans obtained by reductive elimination of bronchial mucins purified from the sputum of 8 CF and 8 CB patients. Our data show that, although the polylactosamine antennae of CF and CB mucins have several terminal sequences in common, they differ significantly in their sialyl Lewis^x (NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAcβ1-) content. Thus all examined mucins from CF patients carry sialyl Lewis^x on their polylactosamine antennae, whereas this type of epitope is present on only three out of the eight CB mucins examined, notably in the airways of one CB patient which were heavily infected by *Pseudomonas aeruginosa* as are the airways of all the CF patients. This suggests that, in airway mucins, the expression of sialyl Lewis^x on polylactosamine antennae is probably more related to inflammation and infection than to a direct effect of the CF defect.

Keywords: **cystic fibrosis, FAB-MS, human airway mucin, sialyl Lewis^x, polylactosamine, airway infection**

Abbreviations: **CB, Chronic Bronchitis, CF, Cystic Fibrosis, FAB, Fast Atom Bombardment; Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid; MS, mass spectrometry.**

Introduction

Human airway mucins represent a very large family of polydisperse high molecular weight glycoproteins which are part of the airway innate immunity. Their peptide part, or apomucin, is encoded by at least six mucin genes. Human airway mucins are highly glycosylated and contain many hundreds of carbohydrate chains which cover the apomucins and add to their structural complexity [1]. This wide carbohydrate diversity yields a repertoire of determinants at the surface of the airways, and suggests that mucins are involved in multiple interactions with microorganisms, thereby playing an important role in the protection of the underlying airway mucosa.

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In some chronic bronchial disorders with a breach in the local defenses, airways may be colonized by *Pseudomonas aeruginosa*, a bacterium which is normally harmless. This is especially the case for patients suffering from cystic fibrosis (CF) where this bacterium is very difficult to eradicate and where the severe infection is responsible for most of the morbidity and mortality observed in this disease.

Cystic fibrosis is due to mutations of a gene localized on chromosome 7 encoding for a membrane glycoprotein, CFTR (cystic fibrosis transmembrane conductance regulator) [2]. CFTR is a chloride channel of low conductance activated by protein kinase A which influences other ion channels and has probably additional unknown functions [3,4]. The link between the CFTR defect and airway infection is unclear. All airway mucins studied so far may interact with *Pseudomonas aeruginosa*, however it has been reported that airway or salivary mucins from patients suffering from cystic fibrosis had an increased affinity for this

bacterium [5,6]. Much attention has been paid to the precocity of lung inflammation in CF patients [7–10], and van Heeckeren et al. have reported an excessive inflammatory response of the airways of CF mice when they are challenged with *Pseudomonas aeruginosa* [11]. More recently, Sajjan et al. have also shown that $Cftr^{-/-}$ knockout mice exposed to repeated instillation of *Burkholderia cepacia* demonstrate an enhanced inflammatory response but apparently less effectively than the $Cftr^{+/+}$ control [12]. Whether or not mucosal features related to an altered inflammatory response in CF pave the way for subsequent bacterial colonization is still an open question.

There is increasing evidence for links between inflammation and glycosylation. Recently, differences in the glycosylation of airway mucins secreted by patients suffering from infected or non-infected bronchial diseases have been observed [13]. Airway mucins may express the Lewis^x epitope as well as sialylated, or/and sulfated derivatives [14–16], and some of these determinants correspond to L-selectin ligands [17]. Increased sialylation and expression of the sialyl-Lewis^x epitope were observed in the mucins secreted by most patients suffering from cystic fibrosis and by a few patients suffering from chronic bronchitis (CB) who have a severe lung infection [13]. These observations raised the question that chronic and severe inflammation of the airway mucosa is responsible for these carbohydrate modifications.

So far, various carbohydrate chains bearing sialyl-Lewis^x determinants have been identified after reductive β-elimination of human airway mucins [14–16] but, due to the limited resolution power of the chromatographic methods available, the chains which have been isolated are relatively short (less than 12 sugars) [1]. There is presently no information on longer chains, such as poly-*N*-acetyllactosamine bearing sialyl Lewis^x, that might represent better ligands for bacteria than the shorter chains.

The aim of the present work was to look for the presence of sialyl Lewis^x epitopes on poly-*N*-acetyllactosamine chains in human airway mucins and to compare their expression in mucins secreted by six severely infected patients suffering from CF and by six patients suffering from CB, who, with a single exception, were without severe infection. For this purpose, purified mucins were submitted to reductive β-elimination. The released *O*-glycans were treated by endo-β-galactosidase digestion, followed or not by reduction with $NaBD_4$, and permethylation/FAB-MS.

The mucins from patients suffering from CF and from one patient suffering from CB, all of them corresponding to severely infected patients, contained more sialyl Lewis^x epitopes on poly-*N*-acetyllactosamine repeats than the mucins from patients suffering from non-infected chronic bronchitis.

Results

Airway mucin purification and estimation of infection

Initial studies optimising our structural analysis methodology were carried out on sputum from two CF and two CB patients. They were completed by studying another series of sputum collected from 6 CF and 6 CB patients.

Airway mucins were purified by two steps of density-gradient centrifugation as previously described [13]. After the first step, the mucin preparations from infected patients were still mixed with DNA that was eliminated before the second step by treatment with nucleases. Before treatment with nucleases, an index of infection was estimated by calculating the ratio: absorbance at 260 nm (nucleic estimation)/absorbance at 520 nm with orcinol (carbohydrate reaction) [13]. The cut-off between infected patients and non-infected, or mildly, infected patients was arbitrarily established at 1.1. The mucin preparations with an index above 1.1 corresponded to severely infected patients, and the mucin preparations with an index below 1.1 were considered as mildly or non-infected patients [13].

The mucins of patients CF1-6 and CB1-6 have been previously studied in detail [13]. Mucins CF1-6 correspond to CF patients (#5, 6, 10–12 of ref [13]); mucin CB2 corresponds to one infected CB patient (patient #15 of ref [13]) with an index of infection at 1.5; and mucins CB1 and CB3-6 which were all considered as secreted by non-infected or mildly infected patients (patients # 30, 31, 33, 36 & 37 of ref [13]), although for CB4, the infection index was border-line, at 1.1. All patients were blood group type O.

All the patients suffering from CF were severely infected by *Pseudomonas aeruginosa* as well as the patient CB4 suffering from chronic bronchitis, in contrast to the other patients suffering from CB.

Structural analysis strategy

In this study we have devised experimental strategies based on endo-β-galactosidase digestion, derivatisation and FAB-MS in order to screen for non-reducing epitopes present on carbohydrate chains containing tandem repeats of *N*-acetyllactosamine. Specifically, mixtures of *O*-glycans, released by reductive elimination from sputum preparations, were subjected, in separate experiments, to the following three experimental protocols: (i) permethylation/FAB-MS; (ii) endo-β-galactosidase digestion followed by permethylation and FAB-MS; (iii) endo-β-galactosidase digestion followed by reduction with NaB^2H_4 and permethylation/FAB-MS.

FAB screening studies on a small portion of the mucins obtained from two cystic fibrosis patients (CF-i and CF-ii) and two chronic bronchitis patients (CB-i and CB-ii) using protocol (i) yielded mass spectra containing in excess of thirty molecular and fragment ions corresponding to glycans up to about ten sugar residues in size. Data from these experiments could be readily interpreted within the framework of the large body of existing structural information pertaining to glycans with short antennae which are known to be present in bronchial mucins. Spectra from samples CF-i and CB-i are reproduced in Figure 1a and b respectively. Samples CF-ii and CB-ii gave spectra similar to Figure 1a and b respectively (data not shown). The molecular and fragment ion compositions (Table 1)

indicate the presence of *O*-glycans rich in sialic acid and fucose. These compositions are fully in accord with earlier rigorous structural studies of bronchial mucins [14–16].

It was not our intention to duplicate published work by characterising the multitude of small to medium-sized *O*-glycans revealed by the FAB screening exemplified by Figure 1. Instead our aim was to take advantage of the mixture analysis capabilities of FAB-MS in an attempt to unambiguously identify the non-reducing sequences present on polylactosamine chains without the need for their separation from the full repertoire of *O*-glycans. We reasoned that this aim could be achieved by comparing data before and after treatment with endo-β-galactosidase. This enzyme cleaves internal β-galactosidic bonds of poly-*N*-acetyllactosamine backbones (see Figure 2 for examples of expected products of endo-β-galactosidase digestion). Thus molecular ions that are observed in the *m*/*z* 500–1500 region of the FAB mass spectra acquired using protocol (ii), which are absent in data from protocol (i), are potentially attributable to digestion products, and, significantly, should include all non-reducing epitopes originally present on polylactosamine chains. Importantly, unlike the bulk glycans present in the initial sample which are alditols as a result of the reductive elimination reaction, the enzymically liberated glycans are not reduced. Therefore we reasoned that it would be possible to confirm assignments by observing any mass shifts resulting from reduction with NaB^2H_4 (protocol (iii)). The predicted mass shift after deuteroreduction is 17 mass units due to the deuterium incorporation plus the formation of an additional methylatable group upon reduction.

Detection of endo-β-galactosidase digestion products

Our first objective was to establish whether polylactosamine-containing glycans were sufficiently abundant in the mucin

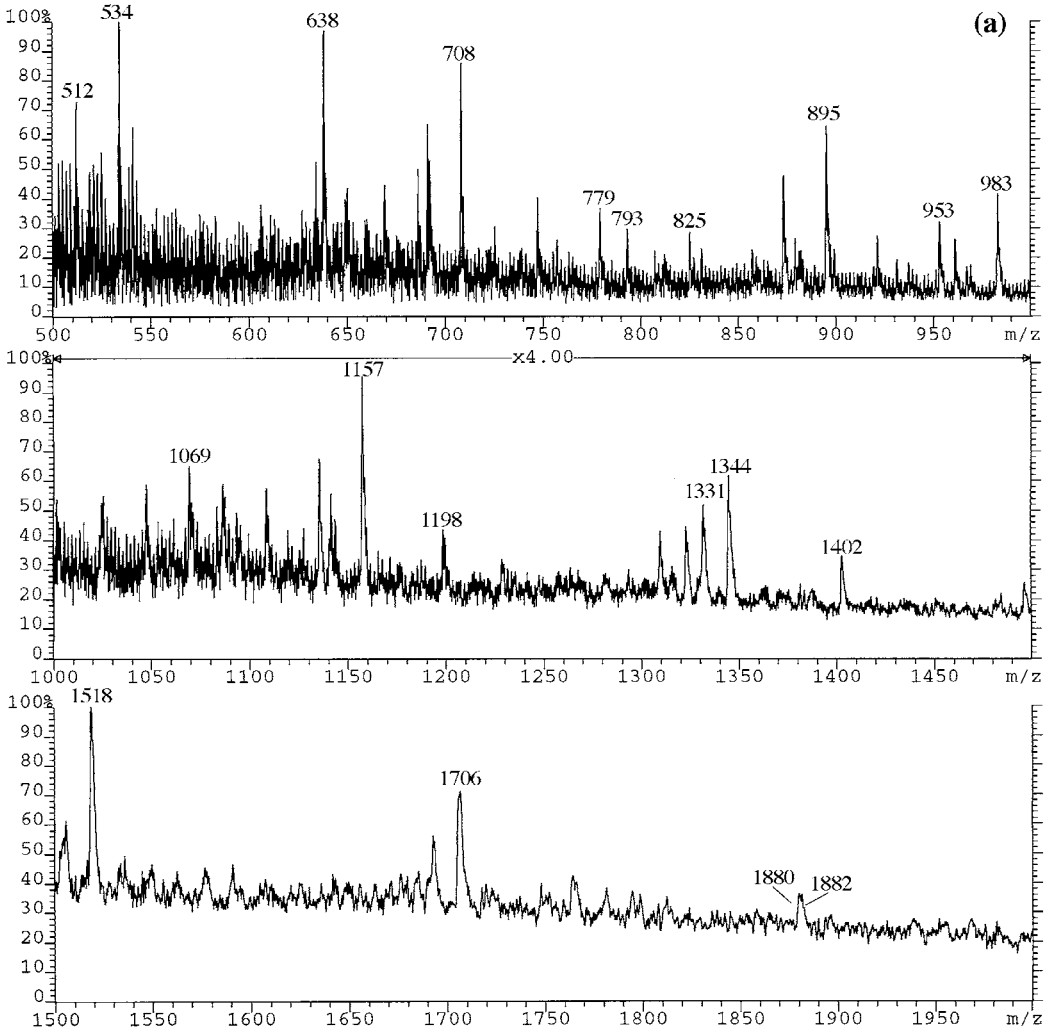

Figure 1. FAB-mass spectrum of permethylated *O*-glycans from bronchial mucins of patients suffering from (a) cystic fibrosis (CF-i) and (b) chronic bronchitis (CB-i). Assignments of molecular and fragment ions are given in Table 1. Annotated peaks not assigned in Table 1 are derived from contaminants.
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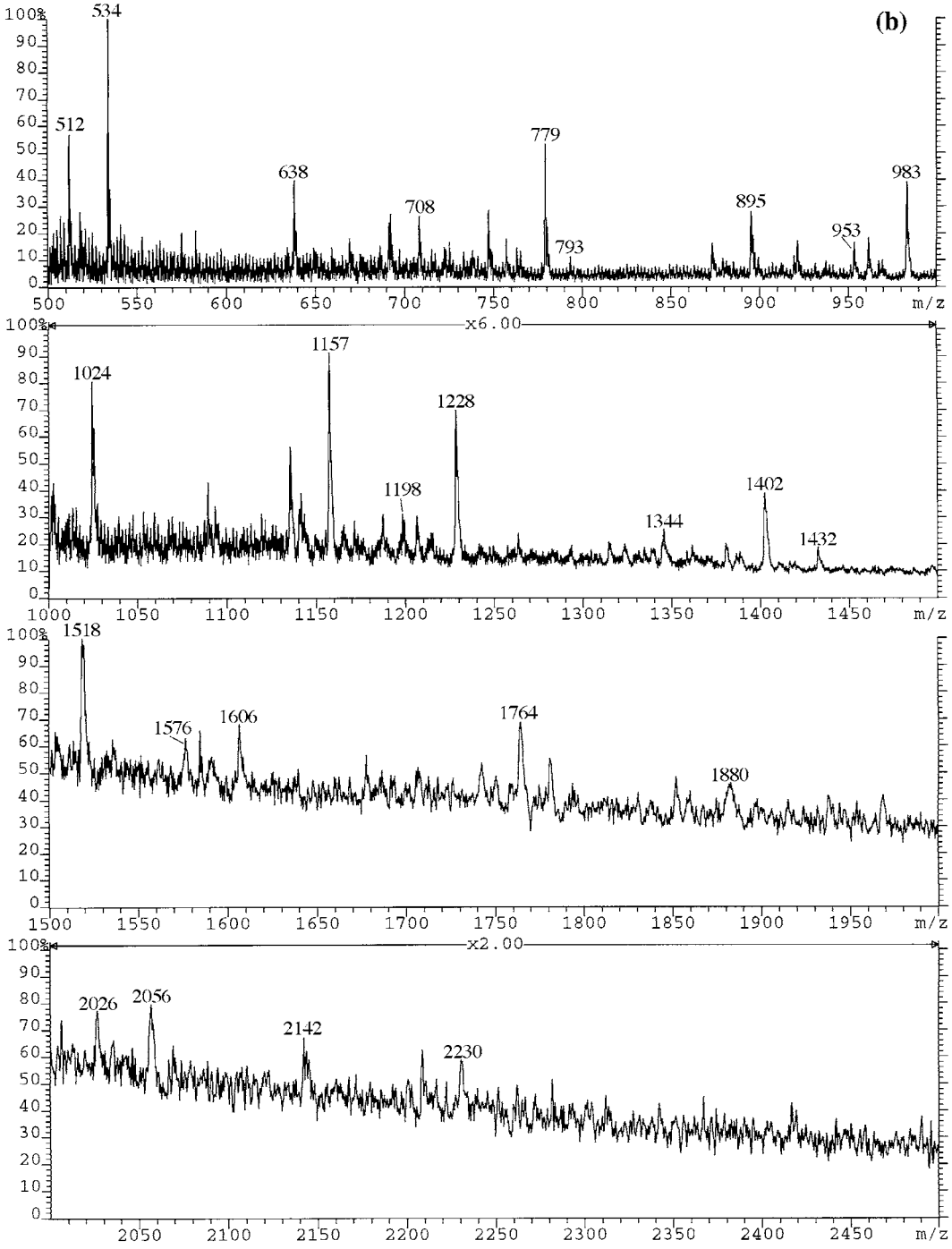


Figure 1. (*Continued*).

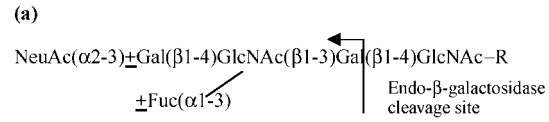
samples to allow the detection of endo-β-galactosidase digestion products in the presence of the multitude of small *O*-glycans released by reductive elimination. Analysis of samples CF-i, CF-ii, CB-i and CB-ii using protocol (ii) provided convincing evidence that digestion products could be detected despite the complexity of the mixtures. Figure 3 shows partial mass spectra spanning the mass range corresponding to non-reducing oligosaccharides that would be liberated from non-substituted and/or sialylated and/or fucosylated epitopes on polylactosamines (see Figure 2 for predicted masses). Comparison with data obtained from analysis of the same mucins using protocol (i) (Figure 1 and data not shown) revealed that new molecular ions are present in all spectra at *m*/*z* 722 (corresponding to Gal-GlcNAc-Gal) and *m*/*z* 896 (corresponding to fucosylated Gal-GlcNAc-Gal). Additionally the cystic fibrosis samples showed signals at *m*/*z* 1083 and 1257 which were weak or

Table 1. Assignments of molecular and fragment ions present in the spectra shown in Figure 1

m/z	*Assignment*	*m/z*	*Assignment*
228	Loss of methanol from 260	1198	$(Fuc_1Hex_1HexNAc_3)$-ol + Na^+
260	$HexNAc^+$	1228	$(Hex_2HexNAc_3)$-ol + Na^+
344	Loss of methanol from 376	1331	$(Fuc_2Hex_2HexNAc_2)$-ol + Na^+
376	$NeuAc^+$	1344	$(NeuAc_1Hex_2HexNAc_2)$-ol + Na^+
432	Loss of methanol from m/z 464 and fucose from m/z 638	1402	$(Fuc_1Hex_2HexNAc_3)$-ol + Na^+
464	$HexHexNAc^+$	1432	$(Hex_3HexNAc_3)$-ol + Na^+
534	$(Hex_1HexNAc_1)$-ol + Na^+	1518	$(NeuAc_1Fuc_1Hex_2HexNAc_2)$-ol + Na^+
638	$FucHexHexNAc^+$	1576	$(Fuc_2Hex_2HexNAc_3)$-ol + Na^+
708	$(Fuc_1Hex_1HexNAc_1)$-ol + Na^+	1606	$(Fuc_1Hex_3HexNAc_3)$-ol + Na^+
779	$(Hex_1HexNAc_2)$-ol + Na^+	1706	$(NeuAc_2Hex_2HexNAc_2)$-ol + Na^+
793	Loss of methanol from m/z 895	1764	$(NeuAc_1Fuc_1Hex_2HexNAc_3)$-ol + Na^+
825	$NeuAcHexHexNAc^+$	1880	$(NeuAc_2Fuc_1Hex_2HexNAc_2)$-ol + Na^+
895	$(NeuAc_1Hex_1HexNAc_1)$-ol + Na^+	1882	$(Hex_4HexNAc_4)$-ol + Na^+
953	$(Fuc_1Hex_1HexNAc_2)$-ol + Na^+	2026	$(Fuc_2Hex_3HexNAc_4)$-ol + Na^+
983	$(Hex_2HexNAc_2)$-ol + Na^+	2056	$(Fuc_1Hex_4HexNAc_4)$-ol + Na^+
1024	$(Hex_1HexNAc_3)$-ol + Na^+	2142	$(NeuAc_1Fuc_2Hex_3HexNAc_3)$-ol + Na^+
1069	$(NeuAc_1Fuc_1Hex_1HexNAc_1)$-ol + Na^+	2230	$(Fuc_2Hex_4HexNAc_4)$-ol + Na^+
1157	$(Fuc_1Hex_2HexNAc_2)$-ol + Na^+		

Also included are low mass fragment ions that are not displayed in the figure.

absent in the chronic bronchitis samples (Figure 3). These signals correspond to sialylated and sialylated/fucosylated structures respectively (see Figure 2). These experiments confirmed that our strategies were capable of revealing epitopes carried on polylactosamine antennae. In addition the data suggested that sialyl Lewis^x sequences were more abundant on polylactosamine antennae of CF mucins than on CB mucins. To explore this further we employed protocols (ii) and (iii) to screen mucins from six additional CF patients (CF1-6) and six additional CB patients (CB1-6) all of whom were blood group type O.



(b)

m/z	Released non-reducing oligosaccharide
722	Gal(β1-4)GlcNAc(β1-3)Gal
896	Gal(β1-4)GlcNAc(β1-3)Gal Fuc(α1-3)
1083	NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal
1257	NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal Fuc(α1-3)

Figure 2. (a) Generic structure of the non-reducing ends of sialylated/fucosylated polylactosamine chains showing the predicted site of endo-β-galactosidase digestion. (b) Predicted non-reducing oligosaccharides which will be generated by endo-β-galactosidase digestion of the sequences shown in (a).

Comparison of endo-β-galactosidase products from six CF and six CB mucins

A total of twelve samples (CF1-6 and CB1-6) were subjected to endo-β-galactosidase digestion and either permethylation/FAB-MS (protocol (ii)) or deuteroreduction followed by permethylation/FAB-MS (protocol (iii)). Mass spectra obtained from CF1 and CB1 are reproduced in Figures 4 and 5 and relevant data from the other ten samples are summarised in Table 2. As observed in the pilot studies on mucins CF-i,ii and CB-i,ii, (see above), all samples displayed signals at m/z 722 and 896 corresponding to unsubstituted and fucosylated non-reducing sequences, respectively (Figures 4a and 5a and Table 2). As predicted, these signals shifted by 17 mass units to m/z 739 and 913, respectively, after deuteroreduction (Figures 4b and 5b and Table 2). The sialylated signal at m/z 1083, which shifted to m/z 1100 after deuteroreduction, was variably present and was more abundant in CF than in CB samples. Importantly all CF samples exhibited a minor molecular ion at m/z 1257 corresponding to sialylated/fucosylated Gal-GlcNAc-Gal together with a fragment ion at m/z 999 resulting from mass spectrometric cleavage at the GlcNAc residue (Figure 4a and b and Table 1). Thus, these data provide evidence for sialyl Lewis^x epitopes being present on the polylactosamine chains of the CF mucins. It should be noted that, although the m/z 1257 signal is co-incident with the ^{13}C-isotope peak of m/z 1256, which is derived from a reduced core type 1 glycan of composition $NeuAc_2HexHexNAcitol$, any ambiguity is resolved by

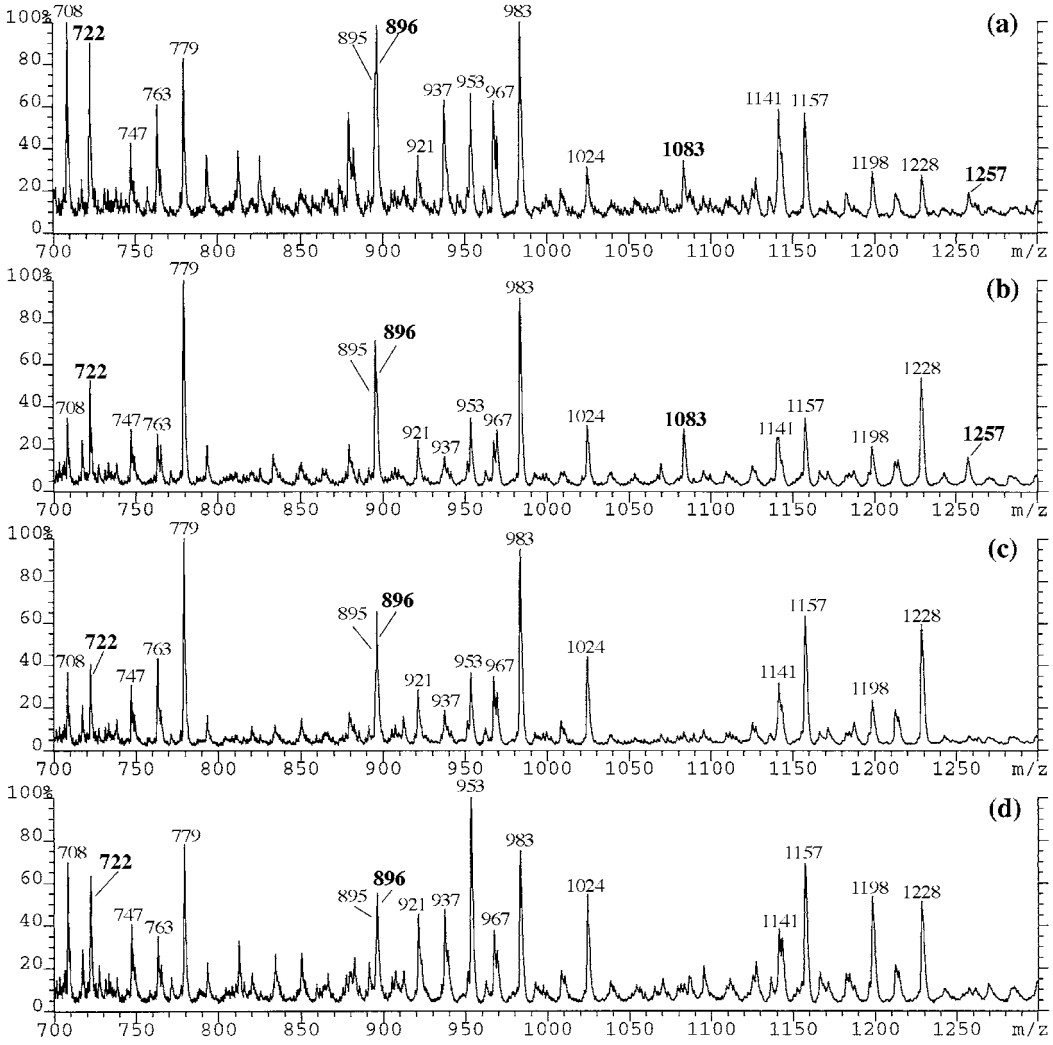


Figure 3. Partial FAB-mass spectra of permethylated *O*-glycans from (a) CF-i, (b) CF-ii, (c) CB-i and (d) CB-ii after endo-β-galactosidase digestion. Signals attributable to digestion products are annotated in bold.

the deuteroreduction experiment which shifts *m/z* 1257 to *m/z* 1274 but leaves *m/z* 1256 and its isotope unaffected (Figure 4b). In contrast to the CF data, the signals at *m/z* 999 and 1257 (or *m/z* 1274 after deuteroreduction) were absent from three CB samples (CB1, 3 and 6) as well as from CB-i and ii (Figures 1b, 5a and b and Table 2).

Discussion

The main objectives of this study were to demonstrate the presence of poly-*N*-acetyllactosamine antennae, which has not yet been proved in airway mucins isolated from patients, to examine the terminal structures carried on these antennae in CF and CB mucins, and, in particular, to establish whether sialyl Lewis^x is a significant epitope on these antennae. This was achieved by comparing FAB mass spectra of mucin-derived glycans before and after endo-β-galactosidase digestion, with and without subsequent deutero-reduction. The molecular and fragment ions obtained prior to endo-β-galactosidase digestion were fully consistent with data from previous studies on CF and CB mucins where glycans carrying short antennae have been rigorously characterised [14–16]. The two major oligosaccharides released by endo-β-galactosidase digestion were derived from unsubstituted (*m/z* 722) and mono-fucosylated termini (*m/z* 896) (see Figure 2). These ions were present in all CF and CB samples. In contrast, the sialyl Lewis^x epitope was observed in the endo-β-galactosidase digestion products of all eight CF samples examined, but in only three of the eight CB samples (CB-2, -4 and -5). With respect to these three CB samples, the strongest data for sialyl Lewis^x structures were obtained from a severely infected patient (CB-2), whilst one of the other patients (CB-4) was considered to be on the borderline of severe infection.

Other workers have shown that oligosaccharides bearing the sialyl Lewis^x epitope on short antennae are present in mucins secreted by CF and CB patients [14–16]. Our study provides

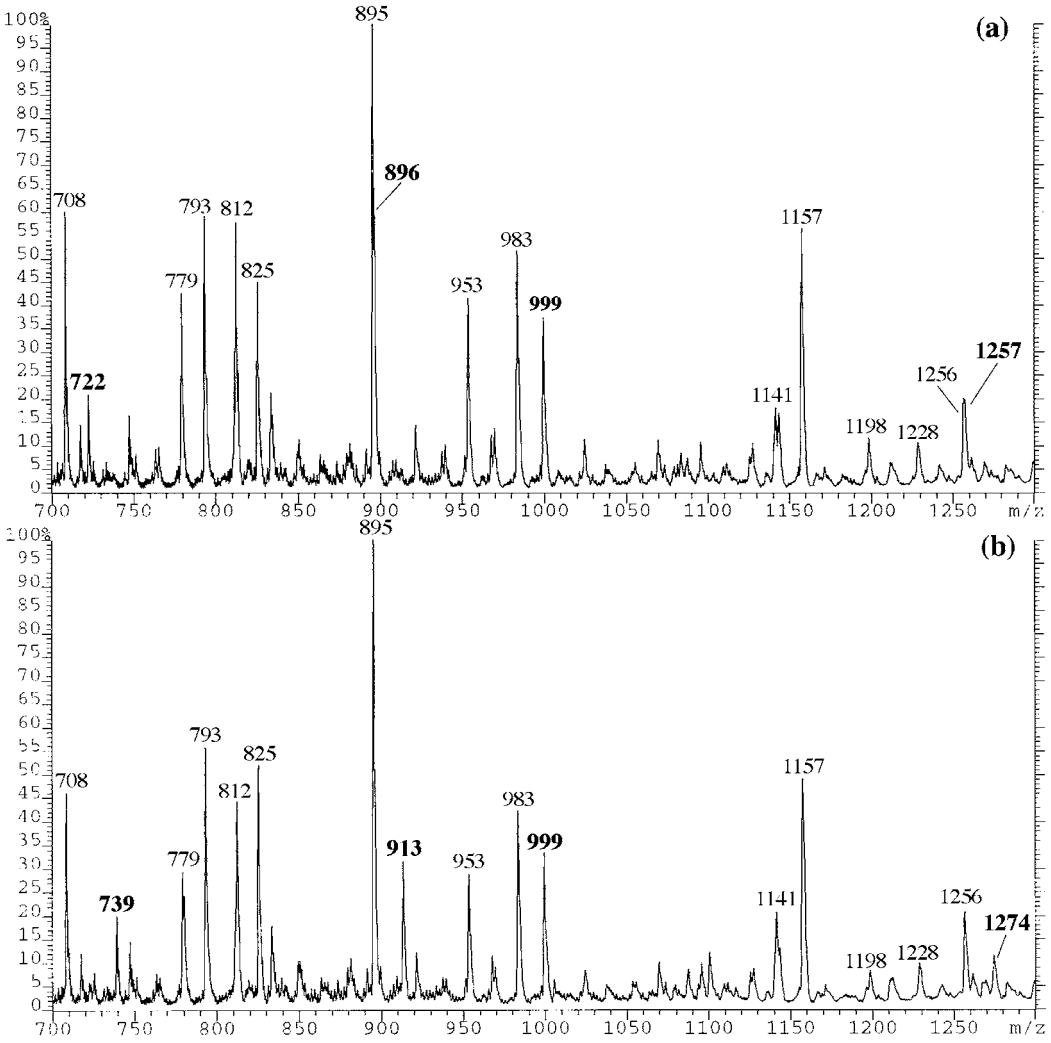


Figure 4. Partial FAB-mass spectra of permethylated *O*-glycans from (a) CF1 after endo-β-galactosidase digestion and (b) CF1 after endo-β-galactosidase digestion and deuteroreduction.

the first evidence for sialyl Lewis^x being attached to poly-*N*-acetyllactosamine antennae in bronchial mucins. In addition we show that this type of structure is more abundant in mucins from severely infected patients such as CF patients than in mucins from non, or mildly, infected patients. Indeed the majority of CB mucins did not contain detectable levels of this epitope in the endo-β-galactosidase digestion products.

Airway mucins secreted *in vivo* are synthesized in goblet cells and mucous cells of the glands that express very little, if any, CFTR [18,19]. Therefore, changes in mucin glycosylation from

Table 2. Presence or absence of predicted products of endo-β-galactosidase digestion of CF1-6 and CB1-6

m/z	*m/z**	*CF1*	*CF2*	*CF3*	*CF4*	*CF5*	*CF6*	*CB1*	*CB2*	*CB3*	*CB4*	*CB5*	*CB6*
722	739	+	+	+	+	+	+	+	+	+	+	+	+
896	913	+	+	+	+	+	+	+	+	+	+	+	+
999	999	+	+	+	+	+	+	−	+	−	+	+	−
1083	1100	+	−	−	+	+	+	−	+	−	−	−	−
1257	1274	+	+	+	+	+	+	−	+	−	+	+	−

The *m/z* values correspond to the structures shown in Figure 2 with the exception of *m/z* 999 which corresponds to the sialyl Lewis^x A-type fragment ion. The *m/z** values are for the deuteroreduced samples.

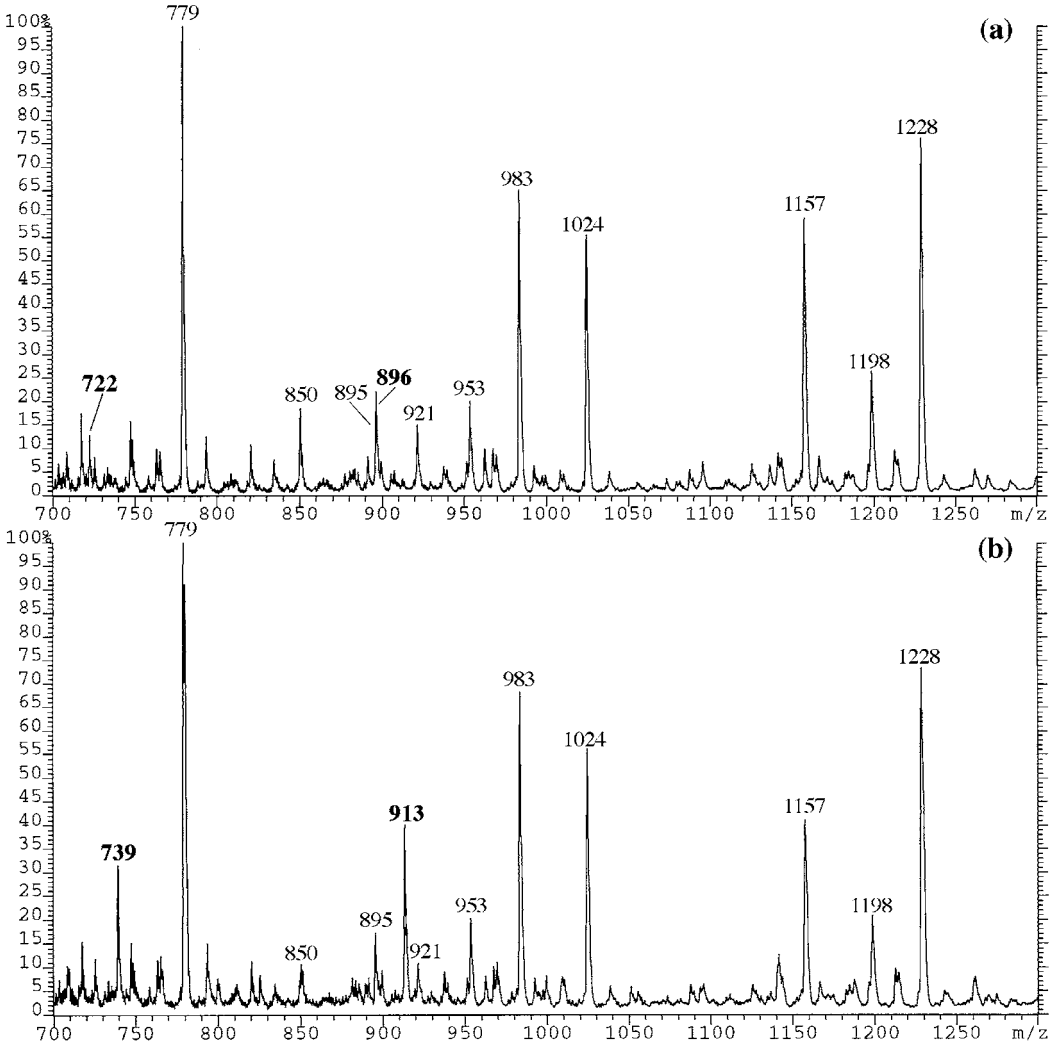


Figure 5. Partial FAB-mass spectra of permethylated *O*-glycans from (a) CB1 after endo-β-galactosidase digestion and (b) CB1 after endo-β-galactosidase digestion and deuteroreduction.

CF patients are probably not related to a direct effect of the CF defect but to a secondary mechanism. As already mentioned, an increased level of sialyl Lewis^x determinants has been observed in mucins from severely infected patients [13]. The reasons for such modifications may be related to the severity of inflammation and infection. In the CF mucosa, several cytokines, such as TNFα, IL-6 or IL-8, may be hypersecreted even before colonization by *P. aeruginosa* [9,10]. Recent observations favour the role of inflammation on glycosylation. For example, in explants of human airway mucosa, TNFα increases the expression of enzymes possibly involved in the biosynthesis of sialyl Lewis^x epitopes: α1,3-fucosyltransferases (FUT3 & 4) and of α 2,3-NeuAc transferases (SATIII & IV) [20]. The present data suggest that inflammation might also influence the expression of the glycosyltransferases responsible for the biosynthesis of polylactosamine chains.

There are many lines of evidence in the literature indicating that *Pseudomonas aeruginosa* binds to airway mucins and to various carbohydrate epitopes [21,22]. For example, using neoglycoconjugates prepared with various Lewis^x derivatives, it was demonstrated that the sialyl Lewis^x determinant was one of the best ligands for different strains of this bacterium and that it could bind to a flagellar protein, FliD, of strain PAO1 that is located at the tip of the flagellum [21]. In the future, it will be interesting to find out if the sialyl Lewis^x on a polylactosamine is a better ligand than the sialyl Lewis^x determinant terminating shorter mucin chains, suggesting that inflammation generates carbohydrate epitopes with a higher affinity for *Pseudomonas aeruginosa*.

Materials and methods

Preparation of airway mucins

Sputum was collected from patients suffering from cystis fibrosis and chronic bronchitis, and kept frozen until used. Mucin

purification was performed as previously described [13]. Briefly, the collected sputa were thawed, diluted with deionised water, stirred overnight at 4°C and centrifuged at 3,000×*g* for 30 min. The supernatants were submitted to a first step of density-gradient centrifugation at 43,000 rpm for 72 h at 10°C. The high-density ($d \geq 1.46$ g/ml) fractions were pooled and exhaustively dialysed against deionized water and freeze-dried. Before lyophilisation, an index of infection was estimated by calculating the ratio: absorbance at 260 nm (nucleic estimation):absorbance at 520 nm with orcinol (carbohydrate reaction) [13]. Then the fractions were treated by DNase and RNase, followed by hyaluronate lyase, chondroitinase ABC, heparinase III from *F. heparinum*, before being submitted to a second step of CsBr density-gradient centrifugation. The high density-, hexose-rich materials which contained the purified mucins were recovered, dialysed and freeze-dried.

Release of oligosaccharides

O-glycans were released from bronchial mucins by reductive elimination (1 M $NaBH_4$ in 0.05 M NaOH, at 45°C for 16 h) and desalted through a Dowex 50W-X8 (form H^+) column. Excess borates were then removed by co-evaporation with 10% acetic acid in methanol under a stream of nitrogen.

Endo-β-galactosidase digestion

This was carried out on released glycans using endo-β-galactosidase from *Bacteroides fragilis* (EC 3.2.1.03, Roche Molecular Biochemicals) in the conditions 0.2 U in 200 μl of 50 mM ammonium acetate pH 5.5. The enzyme digestion was incubated at 37°C for 48 h with a fresh aliquot of enzyme being added after 24 h and terminated by boiling for 3 min before lyophilization.

Reduction with $NaBD_4$

Released glycans after endo-β-galactosidase digestion were reduced with 10 mg/ml $NaBD_4$ in 2 M NH_4OH at room temperature for 2 h and subsequently neutralised with glacial acetic acid and dried under a stream of nitrogen. Excess borates were removed by co-evaporation with acetic acid in methanol (1:10 v:v).

Chemical derivatisation for FAB-MS analysis

Permethylation using the sodium hydroxide procedure was performed as described [23]. After derivatisation the reaction products were purified on C18-Sep-Pak (Waters Ltd) as described [23]. Partially methylated alditol acetates were prepared from permethylated samples for GC-MS linkage analysis as described [24]

FAB-MS analysis

FAB-MS spectra were acquired using a ZAB-2SE 2FPD mass spectrometer fitted with a caesium ion gun operated at 30 kV. Data acquisition and processing were performed using the VG Analytical Opus software. Solvents and matrices were as described [23].

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